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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Null mutation of the galanin gene produced lactational failure following pregnancy. Galanin and galanin receptor 2 (GALR2) are expressed in the mammary gland and are differentially regulated during mammopoiesis. Mammary transplantation experiments demonstrated galanin does not act via autocrine or paracrine mechanisms in the mammary gland. Galanin knockout mice have decreased levels of secreted prolactin and were also found to have an increased ratio of phosphorylated to unmodified prolactin. Unmodified prolactin rescued the lactation failure and treatment of wildtype mice with a molecular mimic of phosphorylated prolactin inhibited lactation and alveolar differentiation. In addition treatment of whole mammary gland explants with galanin resulted in a 3.8 fold increase in the number of lobuloalveoli demonstrating that galanin also has a direct endocrine action on the mammary gland. Investigation of signalling mechanisms showed that galanin activated the Jak/Stat pathway while prolactin acivated the Jak/Stat and MAPK pathways. Galanin was a potent inducer of milk protein gene expression. Transcript profiling revealed the synergist activity of galanin and prolactin. These data identify several novel functions of galanin during mammary gland

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Table of Contents

Galanin, a neuropeptide previously thought to be restricted to the central and peripheral nervous system is implicated in the growth control of a number of cell types. Recently we showed that galanin is expressed by human breast cancer cell lines under the control of estrogen and progesterone. In cell lines derived from small cell lung cancer, a tumour which relapses rapidly with an aggressive phenotype, galanin caused rapid mobilisation of calcium, accumulation of inositol phosphate and activation of the MAP kinase isoform p42 via a protein kinase C dependent mechanism, producing increased clonal cell growth in soft agar. By analogy galanin may also be a growth factor for both the normal breast and breast cancer. This proposal has the objective of testing this hypothesis. The specific aims are to examine the role of galanin in normal mammary gland development, human breast cancer and experimental carcinogenesis.

BODY

The research proposal submitted contains the following objectives (in bold print). Our progress to date with each of these objectives is indicated in plain type. Detailed presentation of our findings can be found in the attached papers and manuscripts.

Specific Aim A: Examination of the role of galanin in normal mammary gland development.

1. **The normal expression patterns of galanin and the galanin receptor subtypes will be examined using *in situ* hybridisation of normal mouse mammary glands at the major developmental stages of puberty, pregnancy, lactation and involution.**

Galanin receptors show a dramatic induction of expression at day 7 of pregnancy that then is rapidly lost, except GalR2 which maintains some expression. Galanin expression is maintained at all times with the exception of involution where it is lost.

2. **The expression pattern of galanin and its receptors by the normal human breast will be examined by *in situ* hybridisation of specimens selected from a panel of 103 breast biopsies obtained at reduction mammaplasty (60 biopsies) or from non involved breast obtained at radical mastectomy (43 biopsies).**

   The production of breast cancer tissue arrays has now been completed after delays caused by the difficulty in obtaining the services of a pathologist. Due to the delay we now have more cancers-203 cancers with greater than 5 years of clinical follow up data have been arrayed. We are now proceeding with this part of the project.

3. **Galanin knockout mice, obtained by collaboration with David Wynick and now at the Garvan Institute, will be used to examine mammary development at puberty, pregnancy, lactation and involution, by whole mount and histological techniques, to determine if galanin is involved in mammary development.**

   These experiments have been completed. A failure of lobuloalveolar differentiation at the late stage of pregnancy was found, resulting in failed lactation.

4. **To determine if the effects defined in A3 are due to the loss of galanin in the mammary gland, or to indirect effects due to the loss of galanin from other endocrine organs,**

animals will be made to endocrinologically normal hosts and examined by whole mount and histochemistry at puberty, pregnancy and involution.

These experiments have been completed. These experiments showed that galanin plays no autocrine role in mammary development- the cause of the defect in galanin kos must lie elsewhere.

Comparison of the recombination and cleared fat pad experiments showed remarkable differences in the level of ductal side branching achieved in virgin animals. The stroma used in these experiments was from 129 animals in the former case and C57BL/6 animals in the later technique. As these strains exhibit different levels of side branching we hypothesised that it was the mammary stromal compartment that underlay this effect, and subsequent experimentation confirmed this.

The failure to transplant the galanin ko phenotype left two possibilities, galanin plays a direct endocrine role, or galanin plays an indirect role. Experiments examining the direct role are detailed below in 5A. The similarity between the galanin ko and the prolactin receptor ko suggest prolactin as a possible indirect mediator of galanin action in the mammary gland. To test this hypothesis we formed a collaboration with Dr. Ameae Walker, UC Riverside, who has supplied us with prolactin and a phosphoprolactin mimic to administer via osmotic minipump. Our results showed that prolactin administration can rescue the first lactation failure in galanin kos, that the phosphoprolactin mimic (S179D) can abrogate lactation in wild type animals, and that galanin knockout mice show altered ratios of

mechanisms of galanin action on the mammary gland appears to reside in the pituitary. First, galanin controls the overall level of prolactin secretion and second galanin controls the phospoprolactin:prolactin ratio, which determines the overall ability of prolactin to modulate mammary development. Although prolactin rescued lactation, it did not rescue milk protein gene expression. We used transcript profiling to examine the transcriptional response to S179D v. prolactin.

5. **The possibility of mammary galanin acting as an endocrine factor to influence mammary development will be examined using conditional galanin knockout animals obtained by collaboration with David Wynick. These animals will have only the mammary gland galanin gene ablated. Hormonal profiles will be measured. A single normal mammary gland will be transplanted to these animals and hormonal profiles again measured. The development of the transplants will be assessed at puberty, pregnancy and involution and compared to a normal mammary glands transplanted to normal hosts.**

The conditional galanin ko animals are not yet available from Dr Wynick. To overcome this we formed a collaboration with Dr. Barbarra Vonderhaar, NCI Bethesda to examine the effects of galanin administration in vitro mammary gland development, using an organ culture technique which has been developed in Dr Vonderhaars laboratory. These experiments showed that galanin exerts a direct effect on mammary gland development. Dr Vonderhaar has supplied us with frozen galanin treated tissue for RNA and protein extraction. As an approved variation in the approved statement of work, we transcript profiled these glands using the Affymetrix U74A2 chips. We have identified sets of genes induced by prolactin

utilised by these hormones. While prolactin activated both Jak/Stat and Map kinase pathways, Galanin was able to activate only the Jak/Stat pathway. The transcript profiling experiments showed that galanin alone could induce milk protein expression (despite not producing alveolargenesis), and this was confirmed by western blot. A very interesting group of genes were identified as regulated by both galanin and prolactin. These experiments showed galanin to be a new systemic hormone involved in mammary gland development. We suspect that the major source of galanin during pregnancy may be placental and that galanin, like prolactin, may be involved in the "hijack" of the maternal endocrine system by the fetus. Galanin would appear to be a hormone inducing terminal differentiation but not cell proliferation of the mammary gland at this stage.

Specific Aim B: Examination of the role of galanin in human breast cancer.

1. **The panel of breast cancer cell lines used to examine galanin gene expression will be screened for galanin receptor subtype expression by PCR. Cell lines selected on the basis of receptor expression will be treated with galanin and cell cycle phase distribution, cell growth and colony formation will be measured.**

Examination of galanin and galanin receptor gene expression in the panel has been completed. In the light of our findings in A above we now expect galanin treatment to reduce or prevent cell growth. Our failure to make cells proliferate with galanin, put down to technical difficulties in previous reports, may have in fact reflected an erroneous assumption that galanin would induce cell proliferation. Rather than using serum free media to slow proliferation

intend to pursue this.

2. **Breast tumours selected on the basis of phenotype from a large panel of specimens will be used to determine which cell types express galanin and its receptors by in situ hybridisation. Results will be related to the normal expression patterns defined in A1 and A2, and to tumour phenotype, steroid hormone receptor expression, markers of poor prognosis, proliferative and apoptotic markers.**

   We are now in a position to proceed with this aspect of the work using our tissue arrays.

3. **The expression of galanin and its receptors by breast cancers will be measured by RT-PCR and correlated with disease outcome in a large panel of breast cancers for which RNA and 74 month average post diagnosis clinical follow-up are available.**

   We are now in a position to proceed with this aspect of the work using our arrays.

Specific Aim C: Examination of the role of galanin in experimental carcinogenesis.

1. **Conditional galanin knockout mice, lacking galanin expression in the mammary glands, will be treated with DMBA, a chemical carcinogen requiring additional hormonal stimulus for full activity. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

   No progress to date. the conditional knockout animals are not yet available

2. **A transgenic mouse expressing galanin under the control of the mouse mammary tumor virus promoter will be constructed and examined for altered rates of tumorigenesis, either spontaneously in virgin and multiparous animals, or in conjunction with DMBA treatment. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

In previous reports we requested to delay this portion of the proposal while we investigated the mechanism of galanin action. We now see galanin as a pro-differentiation hormone and so construction of a galanin transgenic mouse, under an inducible mammary specific promoter is again planned. We will test for the slowing of tumor growth, or the reduction in tumor incidence, following galanin induction.

Discussion

Over the course of this investigation the experimental plan changed as we realised that galanin exhibited a direct action on mammary development. Access to pathology services prevented us from examining galanin in breast cancers until late in the grant period, but this time was occupied with extra experimentation following a line of investigation which unexpectedly revealed galanin to be a novel hormone with a potent differentiative activity in the mammary gland.

The results to date show that galanin plays indirect and direct roles in mammary gland development. The analysis of the role of galanin has proved to be much more time consuming than first envisaged, but our results have shown that galanin's actions are also far more complex than first thought. We have shown for the first time that galanin is a hormone influencing mammary differentiation via multiple mechanisms, which we regard

progesterone and prolactin, the group of circulating hormones directly and indirectly controlling mammary development.

This grant will directly result in the publication of three manuscripts, and has produced data that has been presented in talks at two Gordon Conferences and the Era of Hope meeting.

Key Research Accomplishments

Analysis of the role of galanin in normal mammary gland development has shown that;

•Galanin controls prolactin release from the pituitary and that prolactin administration partially rescues the mammary phenotype in galanin knockout mice,

•Galanin controls the ratio of phosphorylated to non phosphorylated prolactin and that phosphorylated prolactin inhibits lobuloalveolar development,

•Galanin acts directly on the mammary gland to enhance lobuloalveolar development.

•Galanin exerts a differentiative force on the mammary epithelium.

Reportable Outcomes

1. Abstracts of presentations at the following meetings
    a. Australian Society for Medical Research, Leura NSW Nov. 1999
    b. Gordon Conference on Prolactin. Ventura Ca Feb 2000
    c. Keystone Breast and Prostate Meeting, Lake Tahoe NV Mar 2000

2. Oral presentations at the following meetings
    a. Gordon Conference on Prolactin. Ventura Ca Feb 2002

2002

c. DOD Era of Hope meeting. Orlando Fl 2002

3. Manuscripts

a. Naylor MJ and Ormandy CJ (2002) Mouse strain-specific patterns of mammary ductal side branching are elicited by stromal components. *Dev Dyn*. 225, 100-105.

b. Naylor MJ, Ginsburg E, Iismaa, TP, Vonderhaar BK, Wynick D and Ormandy CJ. The neuropeptide galanin is a novel hormone essential for mammary gland development. Submitted.

c. Naylor MJ, Peters K, Ho TWC, Li FC, Walker AM, Wynick D and Ormandy CJ. Galanin controls mammary gland development by regulating prolactin phosphorylation. In preparation.

Conclusions

1.Galanin modulates mammary gland development indirectly via the prolactin system.

So what?

The Nurses Healthy Study (Hankinson 1999) has shown that serum prolactin levels in the top quartile are associated with a 2-3 fold increase in the relative risk of breast cancer, similar to the risk associated with increased serum estrogen levels (Hankinson 1998). The effect of prolactin is independent of estrogen. Thus factors influencing serum prolactin levels can influence susceptibility to breast cancer. Galanin is such a factor.

2. Galanin exerts a direct differentiative activity on the mammary gland.

So what?

We have discovered that galanin is a hormone that acts directly on the mammary gland to induce cell differentiation. Cell differentiation is usually associated with the cessation of proliferation. We may be able to stop tumors growing by exploiting this pathway.

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Hankinson SE. Willett WC. Manson JE. Colditz GA. Hunter DJ. Spiegelman D. Barbieri RL. Speizer FE. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women [see comments]. [Journal Article] Journal of the National Cancer Institute. 90(17):1292-9, 1998 Sep 2.

Appendices

Naylor MJ and Ormandy CJ (2002) Mouse strain-specific patterns of mammary ductal side branching are elicited by stromal components. *Dev Dyn*. 225, 100-105.

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DEVELOPMENTAL DYNAMICS 225:100–105 (2002)

Mouse Strain-Specific Patterns of Mammary Epithelial Ductal Side Branching Are Elicited by Stromal Factors

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ABSTRACT **Variations in mammary ductal side branching patterns are known to occur between different strains of mice and this is related to the rate of spontaneous mammary cancers, which are increased in those strains which show highly side-branched mammary architecture. The cause of the variation in ductal side branching between mouse strains is unknown, but epithelial, stromal, and endocrine factors have been implicated. To define the mammary elements responsible for controlling strain-specific ductal side branching patterns, we formed recombined mammary glands from epithelial and stroma elements taken from highly side-branched 129 and poorly side-branched C57BL/6J mammary glands and transplanted them to Rag1$^{-/-}$ hosts on the inbred C57BL/6J background. When 129 epithelium was recombined with C57BL/6J stroma the poorly side-branched C57BL/6J pattern was observed. C57BL/6J epithelium recombined with 129 stroma resulted in development of the highly side-branched pattern, as did 129 epithelium recombined with 129 stroma. All transplants used the same C57BL/6J endocrine background, demonstrating that strain differences in the mammary stroma are responsible for the strain-specific ductal side branching patterns and that strain differences in epithelium or endocrine background play no part. Genes currently known to influence side branching by means of the stroma include activin/inhibin, epidermal growth factor receptor (EGFR), Wnt-2, Wnt-5a, and Wnt-6. Of these, Wnt-5a mRNA expression was decreased in 129 mammary glands compared with C57BL/6J mammary glands, but in F2 129:C57BL/6J animals Wnt-5a mRNA expression level did not correlate with the highly variable side branching patterns observed. These experiments exclude variation in the expression level of known candidate genes as the mechanism responsible. Regardless of underlying mechanism, transplantation without regard to the genetic background of the stromal donor, whether inbred or mixed, will compromise experiments with side branching and associated gene expression endpoints.** © 2002 Wiley-Liss, Inc.

Key words: mesenchyme; development; tissue recombination; mammary gland; transplantation

INTRODUCTION

Ductal side branching is the formation of ducts by budding from existing ducts. It can be distinguished from the branching produced by bifurcation of the terminal end bud during ductal elongation by the angle that the ducts exit from their parent (near 90 degrees) and their reduced diameter compared with their parent duct. In contrast, branches formed by bifurcation during puberty are Y-shaped and of equal diameter. In virgin animals, ductal side branching occurs during and after puberty and their numbers increase with age in some mouse strains due to the formation of new branches during each estrous cycle (Hennighausen and Robinson, 1998).

Variation in the degree of ductal side branching occurs among different mouse strains. The C3H/HeNHsd, Balb/c, and 129 substrains (Ola, Pas, and SvJ) display a highly side-branched mammary architecture, whereas Nude/onu (nude) and C57BL/6J strains have very little ductal side branching (Gardner and Strong, 1935). The factors controlling this strain variation in mammary side branching are unknown, but as strain-specific branching patterns are also related to the susceptibility of different strains to mammary cancer (Gardner and Strong, 1935; Husby and Bittner, 1946), discovery of the genes involved has direct relevance to breast cancer. These genes may act by means of the mammary stroma, the mammary epithelium, or within the host endocrine system.

The experiments of Sakakura et al. (1976) showed that, when mammary epithelium was recombined with salivary mesenchyme, the architecture of the ductal outgrowth was that of the salivary gland, demonstrating salivary mesenchyme control of mammary epithelial morphogenesis. During pregnancy, the epithelium synthesised α-lactalbumin, a milk protein demonstrating mammary epithelial cytodifferentiation. Later recombination experiments of Cunha et al. (1995) using skin epithelium and mammary mesenchyme showed that, when dorsal or mid-ventral epidermis was com-

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bined with mammary mesenchyme, mammary ducts along with keratinised skin and hair follicles were formed. These experiments demonstrate the inductive power of the mesenchyme over epithelium and indicate that mammary stroma may regulate epithelial side branching. Recent work has identified several factors that act by means of the stroma to control mammary development, such as activin/inhibin, and the epidermal growth factor receptor and ligands (Robinson and Hennighausen, 1997; Cunha et al., 1997; Wiesen et al., 1999).

Epithelial factors may also be responsible for side branching. This hypothesis is supported by recent work that demonstrates a crucial role for progesterone. Progesterone receptor A transgenic mice show a hyperbranched mammary architecture (Shyamala et al., 1998), whereas progesterone receptor knockout mice show an absence of mammary side branches (Lydon et al., 1995) due to the loss of epithelial progesterone receptor (Brisken et al., 1998, 2000). This effect is mediated by means of stimulation of Wnt-4 synthesis, which can act as a paracrine factor, allowing progesterone receptor negative cells to participate in the formation of alveoli (Brisken et al., 2000).

These experiments also indicate that the systemic hormonal environment may influence side branching. Variation in the control of progesterone synthesis by the ovary may exert a role. For example, prolactin regulates progesterone levels, and treatment of prolactin knockout mice with progesterone restores failed ductal side branching (Vomachka et al., 2000). Ductal side branching may also be influenced by estrogen levels (Bocchinfuso et al., 2000) or other hormones that control progesterone receptor levels in the mammary epithelium.

The recent construction of various gene knockout and transgenic models of altered mammary development has led to renewed interest in mammary epithelial and stromal/epithelial recombination transplantation techniques. This interest has facilitated dissection of the signalling pathways and sites of action required for the major events during mammary development (Hennighausen and Robinson, 1998). We have used mammary stroma/epithelial recombination in inbred host animals to determine which of the possible compartments regulates ductal side branching.

RESULTS AND DISCUSSION

Stromal Control of Strain-Specific Mammary Side Branching

Different strains of mice show variations in the level of ductal side branching within the mammary gland. The mice used in this study were the poorly side branched C57BL/6J strain (Fig. 1A) and the highly side branched 129 strain (Fig. 1B). When epithelium from 129 mice was transplanted into $Rag1^{-/-}$ mice, on the inbred C57BL/6J genetic background, by using the cleared fat pad technique devised by DeOme and coworkers (DeOme et al., 1959), the pattern of mammary

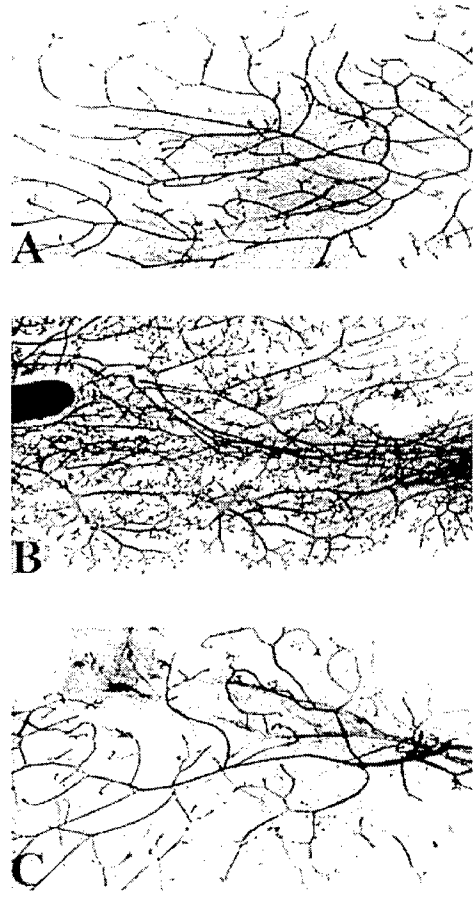

Fig. 1. Whole-mount of 4th mammary gland from 12-week-old mice. **A:** C57BL/6J strain. **B:** 129 Ola/SvPas strain. Whole-mount of mammary epithelial transplant. **C:** 129 Ola/SvPas epithelium/C57BL/6J stroma. Original magnification, ×8.

development observed was that of the endogenous C57BL/6J gland (Fig. 1C). This finding demonstrated that the strain of the stroma or host endocrine environment, but not epithelium, determined side branching patterns. This conclusion, that the strain-specific patterns of ductal side branching are independent of the epithelial component, confirms the work of others using different mouse strains (Yant et al., 1998).

To investigate whether the endocrine environment or the stroma was responsible for controlling the strain differences in side branching, a mammary epithelial/stroma recombination technique was developed. A 1-mm^3 piece of donor tissue (129 or C57BL/6J strain) taken from between the nipple and lymph node of a mature virgin, was inserted into the excised, epithelium-free fat pad from either a 3-week-old 129 or C57BL/6J mouse. The recombined mammary epithelium-stroma complex was then grafted to the abdominal cavity between the 3rd and 4th mammary glands of

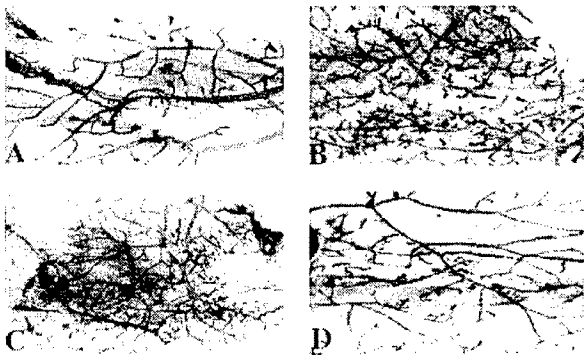

Fig. 2. Whole-mounts from recombination transplants. **A:** 129 epithelium/C57BL/6J stroma. **B:** 129 epithelium/129 stroma. **C:** C57BL/6J epithelium/129 stroma. **D:** Endogenous C57BL/6J mammary gland. Original magnification, ×8.

3-week-old Rag1$^{-/-}$ mouse. By using this technique, we reproduced the previous result; 129 epithelium recombined with C57BL/6J stroma produced poorly branched C57BL/6J architecture (Fig. 2A), whereas recombination of 129 epithelium with 129 stroma resulted in development of the highly branched 129 architecture (Fig. 2B). Similarly, recombinations of C57BL/6J epithelium with 129 stroma produced 129 mammary architecture (Fig. 2C). In all transplants, the endocrine system was inbred C57BL/6J and comparisons were made between transplants grown in the same Rag1$^{-/-}$ animal, allowing us to exclude the endocrine environment as a controlling factor. These studies, shown in Figure 2 and summarised in Table 1, identify mammary stroma as the component responsible for controlling strain-specific ductal side branching patterns. Strain differences in epithelium or the endocrine environment do not cause the differences in ductal side branching between strains.

In mammary recombination transplants, the area of fat pad available for ductal outgrowth is reduced compared with that of the cleared fat pad technique. Transplants of identical combinations of epithelium and stroma were performed by using the recombination and cleared fat pad transplant techniques to investigate whether the level of branching observed in the mammary recombination technique differs due to reduced fat pad area. There was no significant difference in the side branch density of ductal outgrowth from both the transplant techniques and the endogenous gland (Table 1), validating our techniques and confirming our conclusions regarding the controlling role of the stroma.

Investigation of the Role of Stromal Growth Factors Known to Regulate Mammary Ductal Side Branching in Strain-Specific Patterning of the Mammary Gland

Several stromal growth factors have either been identified or implicated in the control of ductal side branching. Stromal activin/inhibin and EGFR signalling are crucial for normal epithelial side branching (Robinson and Hennighausen 1997; Wiesen et al., 1999). Recent work has also demonstrated the role of epithelial expressed Wnt proteins in ductal side branching (Buhler et al., 1993; Bradbury et al., 1995; Brisken et al., 2000). Several members of the Wnt family (Wnt-2, Wnt-5a, and Wnt-6) are also expressed in mammary stroma, where expression precedes ductal outgrowth (Weber-Hall et al., 1994). To investigate whether the level of expression of activin/inhibin, EGFR, or Wnt-2, Wnt-5a, or Wnt-6 may be responsible for the strain-specific differences in ductal side branching, we examined their mRNA levels in the 129 and C57BL/6J mammary glands by using both reverse transcription-polymerase chain reaction (RT-PCR) and quantitative LightCycler real-time PCR. Whole mammary glands rather then glands cleared of epithelium were used to allow epithelial–stromal interactions potentially required for induction of the factors responsible for side branching development. Gene expression was examined at both estrous and diestrous, points of high and low cell proliferation.

No significant difference was observed in activin/inhibin βB subunit or EGFR mRNA expression levels in 129 and C57BL/6J mammary glands during estrous or diestrous by using quantitative PCR (Fig. 3). No consistent changes in mRNA levels of Wnt-2 and Wnt-6 were observed at either estrous or diestrous between the 129 and C57BL/6J mammary glands by using RT-PCR (data not shown). Wnt-5a mRNA expression was found to be 5.1-fold higher ($P = 0.0087$) in C57BL/6J compared with 129 mammary glands taken from mice during estrous and 17.2-fold higher ($P = 0.0196$) in C57BL/6J vs. 129 mammary glands during diestrous (Fig. 3), as determined by quantitative PCR. An inverse correlation between Wnt-5a expression and ductal branching has been noted previously, and down-regulation of Wnt-5a mRNA expression precedes ductal branching (Huguet et al., 1995, Bui et al., 1997).

These data prompted further investigation of Wnt-5a as a possible gene regulating strain-specific ductal side branching. We measured Wnt-5a mRNA levels in a panel of 45 F2 129:C57BL/6J mammary glands. The panel of mammary glands was divided according to their branching phenotype into either high (12 glands) or low (8 glands) ductal side branching density (Fig. 4) and Wnt-5a mRNA levels were measured by quantitative PCR. No difference (1.55-fold difference; $P = 0.39$; Fig. 3) in Wnt-5a expression was found between the two groups, indicating that, although Wnt-5a is differentially regulated in the mammary gland between strains, its expression level cannot account for the difference in ductal side branching patterns.

This investigation allows us to exclude variation in the expression level of known stromal regulators of ductal side branching as the basis for the strain differences observed. Other possibilities remain and include allelic variation or nontranscriptional regulation

TABLE 1. Stromal Control of Ductal Side Branching Density

Experimental condition	Epithelium strain	Stroma strain	Host	n	Side branching density[a]
C57BL/6J control	C57BL/6J	C57BL/6J	C57BL/6J	20	15.3 ± 2.15
129 control	129	129	129	20	54.5 ± 5.86***
Epithelial transplant	129	C57BL/6J	C57BL/6J	9	17.7 ± 2.73
Recombination transplant	129	C57BL/6J	C57BL/6J	7	13.3 ± 1.76
Recombination transplant	129	129	C57BL/6J	8	57.5 ± 6.97***
Recombination transplant	C57BL/6J	129	C57BL/6J	4	54.5 ± 9.50***

[a]Morphometric analysis of mammary transplants. Quantification of ductal side branching was performed by counting the number of side branches per 10× field. Four representative fields of view from each gland were counted. Results are expressed ± SEM.
***$P < 0.0001$ vs. C57BL/6J control gland.

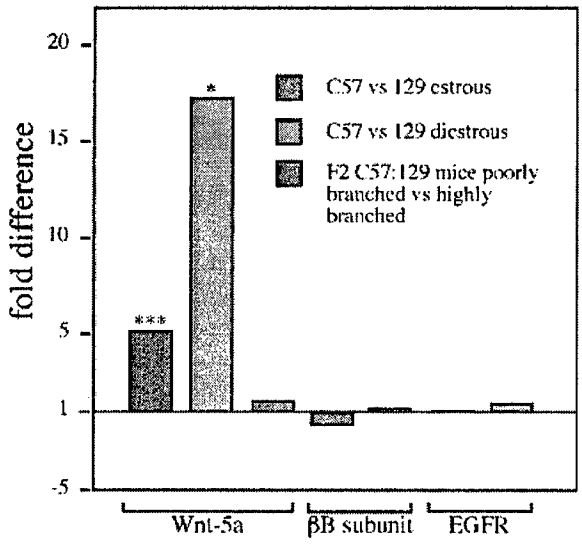

Fig. 3. Quantitative polymerase chain reaction for activin/inhibin βB subunit, epidermal growth factor receptor (EGFR), and Wnt-5a mRNA levels in 129 and C57BL/6J mammary glands at estrous or diestrous as indicated. F2 129:C57BL/6J (F2 C57:129) mammary glands were separated into either poorly or highly side-branched phenotypes on the basis of morphology. Relative fold difference of mRNA levels between groups is indicated and determined as described in text. *$P = 0.0196$, ***$P = 0.0087$

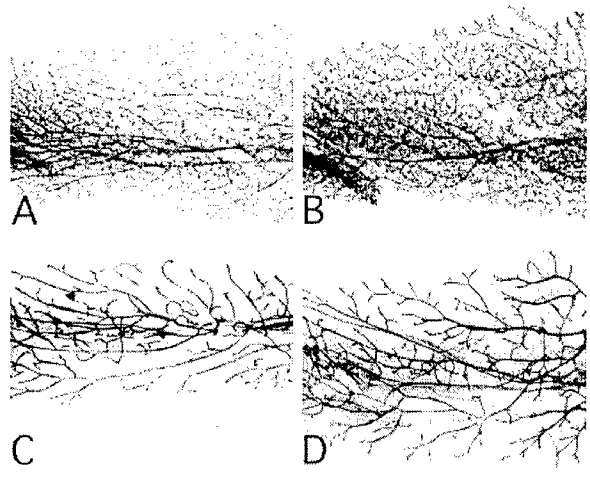

Fig. 4. Variation in ductal side branching between F2 generation 129:C57BL/6J littermates at 16 weeks of age. **A,B:** Highly side-branched mammary architecture similar to the 129 strain. **C,D:** Poor ductal side branching characteristic of the C57BL/6J strain. Original magnification, ×8.

among these candidate genes or as yet to be identified genes. Study of inheritance pattern coupled to a quantitative trait loci approach may shed some light on the genetic basis of this observation.

Strain Variation in 129:C57BL6/J Mice Potentially Complicates Interpretation of Mammary Transplantation Experiments

We examined the ductal side branching density of 45 F2 generation 129:C57BL/6J mice. Figure 4 demonstrates the side branching variability observed in F2 generation 129:C57BL/6J outbred mice. Of the 45 mice examined, 12 demonstrated the highly side-branched pattern of the 129 strain (Fig. 4A,B), whereas 8 displayed the very poor side-branching pattern characteristic of the C57BL/6J strain (Fig. 4C,D). The remaining 25 mice demonstrated side-branching patterns that were intermediate between the two extremes.

Transplantation and mammary recombination techniques have become an invaluable tool in the elucidation of the role of various components controlling mouse mammary gland development, especially with the adoption of transgenic and knockout techniques. Several mammary transplantation techniques have been described depending on the nature of the genetic alteration and the questions being addressed (DeOme et al., 1959; Cunha et al., 1997; Brisken et al., 1998). Use of immunocompromised mice allows transplantation between dissimilar mouse strains, and $Rag1^{-/-}$ mice serve as a good host system as they have a normal endocrine environment (Brisken et al., 1999) compared with nude mice (Kopf-Maier and Mboneko, 1990). This study demonstrates the importance of considering the genetic background of host or stroma-donor animals. Two potential problems may arise. The use of stroma

from a mouse strain that exhibits poor side-branching will limit the degree of possible side branching obtained, masking genetically induced changes in side branching morphology. Second, transplantation by using a mixed genetic background stroma, such as a mixed 129:C57BL/6J Rag1$^{-/-}$ (the strain currently commercially available) will result in highly variable ductal side branching, again obscuring an effect on side branching.

CONCLUSIONS

By using mammary gland recombination and transplantation, we have demonstrated that the stroma, regardless of the strain of epithelium or endocrine environment, controls the differences in mammary epithelial patterning observed between different strains of mice. The expression levels of genes known to influence ductal side branching by means of the stroma are not responsible. Unknown genes, allelic variation, multiple genes, or nontranscriptional mechanisms may be responsible. Correct choice of the strain of host stroma is crucial to the correct interpretation of mammary transplantation experiments.

EXPERIMENTAL PROCEDURES

Mice

All 129 mice used in these experiments were the 129 Ola/SvPas mixed substrain. Rag1$^{-/-}$ mice (Mombaerts et al., 1992) of the C57BL/6J strain were purchased from Animal Resource Centre, Perth, Australia. All animals were housed with food and water ad libitum with a 12 hr day/night cycle at 22°C and 80% relative humidity.

Mammary Epithelium Transplants

Mammary epithelium transplants were performed as previously described (DeOme et al., 1959). In brief, an approximately 1-mm^3 section of mammary gland was excised from 12-week-old 129 or C57BL/6J strain donors from between the nipple and lymph node of the 4th mammary gland. The opposite 4th mammary gland was taken for carmine-stained histologic examination as a reference. The donor mammary tissue was transplanted into the cleared mammary fat pad of 3-week-old Rag1$^{-/-}$ mice. Epithelium from the transplanted tissue grows out into the host fat pad forming a new association with the host stroma. The transplanted gland and an endogenous gland were examined by histology or whole-mount analysis 12 weeks after surgery, at this time an entire functional mammary gland had developed. Twenty-seven of 28 (96.4%) transplants performed successfully engrafted. Morphologic examination of the formed glands showed that the observed ductal outgrowth was always derived from the transplanted epithelium and never from endogenous epithelium. Ductal outgrowth resulting from transplanted epithelium originates from the centre of the gland, whereas a ductal structure originating from the edge of the gland indicates outgrowth that is derived from endogenous epithelium (Lewis et al., 1999).

Recombined Mammary Gland Transplantation

Transplantation of recombined mammary glands were performed by inserting donor tissue (129 or C57BL/6J strain) prepared as described above, into the excised fat pad of either 129 or C57BL/6J 3-week-old mice cleared of endogenous epithelium. The recombined mammary epithelium-stroma complex was then, at the same time, grafted between the abdominal cavity and skin, placing the transplant between the 3rd and 4th mammary glands of 3-week-old Rag1$^{-/-}$ mice (Brisken et al., 1998). The following recombinations of mammary epithelium (Ep) and stroma (St) were performed: 129 Ep & C57BL/6J St; 129 Ep & 129 St; C57BL/6J Ep & 129 St; and C57BL/6J Ep & C57BL/6J St. Recombined transplanted mammary glands were examined by whole-mount histology at 6 weeks after surgery. We found the recombination technique to have engraftment success rates similar to that of the epithelial transplant technique, with 24 of 26 (92.3%) transplants performed having successfully engrafted.

Histology

Mammary whole-mounts were performed by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining overnight. The whole-mount was dehydrated by using a graded ethanol series followed by xylene treatment for 60 min and storage in methyl salicylate (Bradbury et al., 1995).

Morphometric Analysis

Quantification of ductal side branching was performed by counting the number of side branches (branches formed by bifurcation were not included in count) per 10× field. Four representative fields of view from each gland were counted in triplicate by at least two independent researchers. Results are expressed ± SEM.

mRNA Isolation

The 4th inguinal mammary gland was used for mRNA isolation. Upon removal, the gland was placed in liquid nitrogen before storage at −80°C before use. Total RNA was extracted by using TRIZOL Reagent (Gibco BRL, NY) according to the manufacturer's instructions. The stage of estrous cycle was determined after a vaginal smear by using Diff Quick Stain (Lab Aids, NSW, Australia).

Quantitative PCR

First strand cDNA synthesis was performed by using avian myeloblastosis transcriptase (Promega, WI), according to the manufacturer's instructions. Quantitative PCR was performed by using LightCycler technology (Roche, Mannheim, Germany). PCR primers for

Wnt-2 (Acc. no. AF229843), Wnt-5a (Acc. no. NM009524), Wnt-6 (Acc. no. NM009526), activin/inhibin βB subunit (Acc. no. X69620), EGFR (Acc. no. AF275367), and GAPDH (Acc. no. M32599) were designed on the basis of mismatch to other genes. The following primers were used in this study: (Wnt-6)mWnt6F1 5′-ATGGGCTTTCGGGTTCCTG-3′ (forward) and mWnt6R1 5′-CGCAGAAGTCGGGTGAATCG-3′ (reverse), (Wnt-5a) mWnt5aF1 5′-ACAATACTTCTGTCTTTGGCAGGG-3′ (forward) and mWnt5aR1 5′-TACTTCTCCTTGAGGGCATCGC-3′ (reverse), (Wnt-2) mWnt2F15′-TCTGGCTCCCTCTGCTCTTGAC-3′ (forward) and mWnt2R1 5′-TGGTGATGGCAAATACAACGC-3′ (reverse), (βB subunit) βBF1 5′-GTGGTGGGAAAGTTTGGAGGTAG-3′ (forward) and βBR1 5′-GGGTAAAAAAGCCGCACAATC-3′ (reverse), (EGFR) EGFR-F1 5′-TGGTCAGTTTTCTTTGGCGG-3′ (forward) and EGFR-R1 5′-ACATTCTGGCAGGAGACACAGTCC-3′ (reverse). PCR reactions were optimised for LightCycler conditions by titrating MgCl concentrations and annealing temperatures. PCR reactions were performed in 10-μl volume with 1 μl of cDNA, 5 pmol of each primer and FastStart DNA Master SYBR Green I enzyme mix (Roche, Mannheim, Germany), according to the manufacturer's instructions. Relative quantification of the product was performed by comparing the crossing points of different samples normalised to an internal control (GAPDH). Each cycle number in the linear phase of the reaction corresponds to a twofold difference in transcript levels between samples. Each reaction was performed in triplicate by using a minimum of four samples and by using cDNA from at least two separate cDNA synthesis reactions. Statistical significance was determined by using Student's unpaired *t*-test.

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The neuropeptide Galanin is a hormone essential for mammary lobuloalveolar differentiation.

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Abstract

Null mutation of the galanin gene has shown it to be essential for neuronal development and regulation of pituitary prolactin secretion correlated with failed lactation. But galanin is also expressed by the mammary gland, leading us to investigate the mammary action of galanin in detail. We report that although prolactin supplementation of galanin knockout mice enabled pup survival, lobuloalveolar differentiation remained impaired. In organ culture addition of galanin directly induced epithelial differentiation, but not proliferation, via activation of the Jak/Stat pathway. Supplementation with prolactin and galanin, activated both Jak/Stat and Map kinase pathways and resulted in lobuloalveolar growth and differentiation far beyond that achievable with prolactin alone. Examination of gene expression patterns revealed overlapping, unique and synergistic patterns of transcriptional activation by these hormones. These data establish a new role for galanin as a hormone essential for lobuloalveolar differentiation during pregnancy.

Introduction

Postnatal development of the murine mammary gland is systemically controlled by the pituitary-ovarian axis. Each of these hormones control different facets of mammary development, for example, estrogen and growth hormone regulate ductal elongation and bifurcation, progesterone is essential for ductal side branching and alveolar bud formation, while prolactin is critical for lobuloalveolar development and lactogenesis. It is now also apparent that these hormones induce several common signalling pathways that, in turn, direct epithelial morphogenesis (Hennighausen and Robinson, 2001).

Galanin is a 29 amino acid peptide originally isolated from porcine intestine (Tatemoto et al., 1983) that has been implicated in the control of a number of biological processes including cognition, feeding behaviour, neuroendocrine responses, mitogenesis and nociception (Iismaa and Shine, 1999). Galanin signals through a family of three G protein-coupled receptors, galanin receptors (Galr) 1-3 (Habert-Ortoli et al., 1994; Howard et al., 1997; Wang et al., 1997). The generation of mice carrying a loss-of-function mutation of the galanin gene has enabled investigation into the functions of galanin *in vivo* where it regulates the development of sensory and cholinergic neurons (Holmes et al., 2000; O'Meara et al., 2000). Galanin is also a growth factor for the prolactin-secreting pituitary lactotroph and galanin knockout mice display reduced prolactin levels during pregnancy which correlated with lactational failure (Wynick et al., 1998). Overexpression of galanin in the lactotroph induces hyperprolactinemia and pituitary hyperplasia (Cai et al., 1999).

Galanin also acts as a mitogen for a number of small cell lung cancer cell lines, (Sethi and Rozengurt, 1991a; Sethi and Rozengurt, 1991b). The galanin gene is located at 11q13, and like many genes in this region it is amplified in around 13% of breast tumours (BCRT rev). Galanin is expressed by a number of breast cancer cell lines, but expression does not correlate with amplification. In contrast, galanin expression correlates with estrogen and progesterone receptor expression and is regulated by estradiol and progesterone (Ormandy et al., 1998). This observation suggested that galanin's role in mammary gland development may involve more than simple modulation of pituitary prolactin production. We have utilised galanin knockout mice, combined with mammary transplantation, whole organ culture and transcript profiling to examine in detail the role of galanin in mammary development. Galanin was found to act directly on the mammary gland to induce epithelial differentiation, and in concert with prolactin to enhance the growth of the lobuloalveoli well past that produced by prolactin alone. These data establish galanin as a new hormone regulating epithelial cell differentiation during mammopoiesis.

Results

Galanin and galanin receptors are differentially expressed in the mammary gland

Expression of galanin and Galr 1-3 was examined by RT-PCR using mammary glands collected at various stages of development (Fig. 1). Galanin was expressed at all time points from estrous in virgin mice through to lactation, but significantly was not detected during involution. Galr expression was tightly regulated and cooridinated. All three receptors were most highly expressed at day 7 of pregnancy. Galr1 was only detected at this time, Galr2 was also detected at lower levels throughout the later stages of pregnancy and involution, and Galr3 mRNA was also detected during estrous and diestrous. Very low expression of Galr3 could also be detected at 5 days of involution with longer exposure (data not shown).

Prolactin rescues lactational failure in $Gal^{-/-}$ mice, but fails to completely restore lobuloalveloi differentiation

Targeted disruption of the galanin gene results in failure of ductal side branching during puberty and lactation failure following pregnancy (Wynick et al., 1998) but mammary development during pregnancy has not been investigated. At day 12 of pregnancy the amount of alveolar development was decreased in $Gal^{-/-}$ mammary glands compared to galanin wild-type ($Gal^{+/+}$) mice (Fig. 2A). This defect in alveolar profileration continued throughout pregnancy and at the 1st day post-partum $Gal^{-/-}$ mammary glands showed reduced lobulo-alveolar development compared to the normal development observed in $Gal^{+/+}$ mice (Fig. 2B). Histological examination showed that lactation had not

commenced in $Gal^{-/-}$ mice, where small alveoli showed colostrum retention (Fig. 2C). Differentiation of the mammary epithelium was assessed by quantitative analysis of the mRNA levels of several milk protein genes. Early (WDMN-1), mid (β-casein) and late (WAP) stage markers of epithelial cell differentiation were all decreased in $Gal^{-/-}$ mammary glands compared to $Gal^{+/+}$ littermates (Fig. 2D) confirming the morphological and histological observations. Examination of the stomach contents of pups showed that 11 of 12 knockout females were unable to lactate following their first pregnancy (Fig. 2F), despite the observation of normal maternal behaviour and suckling of pups. Interestingly this effect was lost following their second pregnancy.

Since homologous disruption of the galanin gene results in decreased levels of plasma prolactin, we examined whether treatment of $Gal^{-/-}$ mice with prolactin would rescue the defect in lobuloavleolar development and subsequent lactation failure. Treatment with either 0.6 or 1.2 μg of prolactin per 24 hours throughout the duration of pregnancy restored lactation to a point sufficient for pup survival (Fig. 2F). Whole mount investigation demonstrated restoration of lobuloalveolar development comparable to wild type mice (Fig. 2B), but histological examination showed that compared to wild type animals there were many more ducts that had not commenced lactation and which retained colostrum (Fig. 2C). Analysis of milk protein gene expression revealed that prolactin treatment completely failed to rescue the defect in mammary differentiation revealed by the expression of the milk protein genes (Fig. 2E). Thus, although prolactin treatment restored development and lactation to a level sufficient for pup survival, it did not completely restore mammary differentiation to wild type levels.

Galanin does not act via an essential autocrine or paracrine mechanism to regulate mammary development

The incomplete rescue of lobuloalveolar development following prolactin treatment in $Gal^{-/-}$ mice indicated that galanin must act by an additional mechanism to regulate epithelial cell development. The secretion of galanin by the pituitary and coordinated regulation of galanin receptors in the mammary gland during pregnancy suggests a possible endocrine role for galanin, while the expression of galanin in the mammary gland also raised the possibility of an autocrine or paracrine mechanism. To determine whether mammary galanin production is essential for normal development, recombined glands were formed in which the galanin gene was lacking in either the mammary epithelium or stroma, on a background of normal host endocrinology, including circulating galanin levels. Deletion of galanin from the stroma or from the epithelium, or from both, did not alter mammary architecture observed during puberty or on the 1st day post-partum (Fig. 3 and data not shown). These data demonstrate that mammary galanin production is not essential for normal mammary morphology or histology.

Galanin can act directly on the mammary gland to induce alveolar differentiation and proliferation

Next we determined if circulating galanin could act directly on the mammary gland to induce ductal side branching or alveolar proliferation and differentiation. As galanin treatment *in vivo* may indirectly induce mammary development via endocrine regulation, we utilised an *in vitro* mammary gland explant model of mammogenesis (Plaut et al., 1993).

Ductal side branching similar to that seen during puberty was produced when mammary gland explants were cultured in insulin (I), aldosterone (A) and hydrocortisone (H) (Fig. 4). The addition of 100 nM galanin to the medium did not alter ductal development measured by quantitative morphology and histology. When prolactin was added to the culture medium, lobuloalveolar development was observed (Fig. 4), though as noted previously not to the extent observed during pregnancy *in vivo*. The addition of 100 nM galanin to IAH-Prolactin medium resulted in a 3.8 fold increase in the number of lobuloalveoli per gland (8.6±2.1 IAH+prolactin v. 33.0±6.1 IAH+prolactin+galanin, P=0.005), causing the glands to resemble those observed *in vivo* during pregnancy. Additionally, the size of individual lobuloalveoli in IAH+prolactin+galanin treated glands was also greater than in IAH+prolactin treated glands alone (Fig. 4). These data show that galanin can act directly on the mammary gland to augment prolactin-mediated lobuloalveolar development, establishing galanin as a new endocrine factor active during this phase of development.

Galanin and prolactin induced signalling pathways in the mammary gland

We next investigated activation of the Jak/Stat, MAP kinase and PI3 kinase signalling pathways by prolactin and galanin. As expected, in explants treated with prolactin we saw activation of the Jak/Stat pathway, with an increase in total Stat5 and a dramatic increase in phosphorylated Stat5 in these samples (Fig. 4). Similarly, and as also expected, prolactin treatment activated the MAP kinase pathway, with the level of total ERK1/2 decreased and the levels of phosphorylated ERK increased in explants receiving prolactin. Examination of the AKT pathway revealed decreased mobility but no increase

in total AKT in explants receiving prolactin, but no increase in phosphorylation of the two residues most important to AKT activation. The decrease in mobility may represent phosphorylation of other sites on the AKT molecule.

Suprisingly, galanin alone was able to activate the Jak/Stat pathway, similar to prolactin (Fig. 4). In stark contrast to prolactin, galanin did not induce activation of the MAP kinase pathway or alter the mobility of AKT. When explants were treated with galanin and prolactin there were no dramatic changes to the effects produced by either hormone alone. The apparent slight dimunition in pERK and increase in AKT and pAKT(T308) were not consistent between experimental replicates.

We examined makers of mammary epithelial cell differentiation by western blot. Again as expected, explants treated with prolactin showed synthesis of the milk proteins WAP and alpha and beta casein. Strikingly, galanin alone produced the greatest induction of milk protein synthesis (Fig. 4).

Together these results show that galanin, via sole activation of the Jak/Stat pathway can induce epithelial cell differentiation, measured by milk protein synthesis, in the absence of epithelial cell proliferation, measured by lobuloalveolar development. In contrast prolactin, which activates the Jak/Stat, MAP kinase and possibly PI3 kinase pathways, produces both epithelial cell differentiation and epithelial cell proliferation. Together these hormones allow lobuloalveolar development to proceed *in vitro* to a level beyond that achievable by prolactin alone, and thus further than lobuloalveolar differentiation has previously been taken *in vitro*.

Transcript profiling of galanin and prolactin induced mammary development

We next examined the transcriptional response of the mammary gland to galanin and prolactin using the Affymetrix cDNA microarray system.

The total number of genes found either increasing or decreasing greater then 1.7 fold compared to IAH treatment alone were determined using Affymetrix GeneChip v5 software (MAS 5). In addition, in all analysis a gene was considered regulated by a particular treatment/s only if in the other treatment groups not being compared the gene also had less than a 1.2 fold change in the same direction, regardless of the presence and absence calls. This method greatly strengthened the confidence that a gene was only regulated by a particular treatment and was not simply below the level of detection as determined by MAS 5. This analysis (Fig. 5) was validated using quantitative RT-PCR and similar results were also found using ANOVA, ranking by the coefficient (data not shown).

It is immediately apparent that the vast majority of genes regulated in response to galanin and prolactin are divided into two groups (Fig. 5A), those regulated in common by galanin and prolactin (galanin, prolactin and galanin + prolactin) and those regulated only by prolactin (prolactin, prolactin + galanin but not galanin alone). These groupings potentially reflect two different cell fate processes in the mammary gland. Examination of the genes regulated in common between galanin and prolactin identify genes that are consistent with mammary epithelial cell differentiation such as the milk proteins (WAP, WDMN-1 and 5 casein family members). Interestingly galanin in the absence of prolactin produced the greatest induction of the milk protein genes. This group of genes also includes regulators of the Jak/Stat signalling pathway (CIS, SOCS2) consistent with our finding that galanin activates this pathway. A number of genes essential for mammary

gland development are also regulated by both galanin and prolactin including E74-like factor 5 (Elf5), growth hormone receptor (GHR), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 5 (IGFBP5), helix-loop-helix protein Id2 and osteopontin. These data demonstrate that galanin and prolactin regulate a common and central pathway to induce epithelial cell differentiation. In addition prolactin regulated a group of genes not regulated by galanin alone. This group of genes must represent proliferation induced genes as galanin alone was unable to induce epithelial cell proliferation.

The next largest group of genes were those regulated by just galanin. Interestingly, only 5 of these genes were also regulated by prolactin + galanin, indicating that most of these genes are inhibited by prolactin, they may represent genes required for some other as yet unidentified function of galanin in the mammary gland or are differentiation genes lost following prolactins induction of proliferation. A small subset of 14 genes were regulated by just prolactin + galanin but not either hormone alone identifying a synergistic effect of these two hormones.

Overall these data show that galanin is essential for lobuloalveolar development by a direct endocrine mechanism, establishing galanin as a novel mammary gland hormone.

Discussion

This study identifies several novel functions of galanin during mammary gland development. Galanin acts as a positive regulator of prolactin signalling via control of prolactin secretion and in addition to these indirect effects, galanin acts directly on the mammary gland to induce epithelial cell differentiation and to augment the prolactin-initiated process of alveolar proliferation.

These findings establish galanin as an important modifier of prolactin's actions in the mammary gland, and as a new hormone directly involved in lobuloalveolar development. These actions are summarised in Fig 6. Galanin regulates pituitary prolactin secretion (Wynick et al., 1998). Work with Prlr knockout mice has defined the action of prolactin. It acts indirectly during puberty to control ductal side branching via regulation of ovarian progesterone (MJN and CJO unpublished). Estrogen levels are also decreased in Prlr knockout mice but not sufficiently to alter ductal elongation and bifurcation. In mammary transplants from this model, development stalls during pregnancy following alveolar bud formation, demonstrating that signalling via the mammary epithelial Prlr is essential for lobuloalveolar development (Brisken et al., 1999).

In addition to controlling mammary development via regulation of prolactin, galanin also acts directly on the mammary gland to induce lobuloalveolar development. Galanin probably exerts its greatest direct influence on mammary development during early pregnancy, at the onset of alveolar development, as this is when all three receptors are expressed and expressed at their highest levels. Serum galanin levels start to rise at day 7 of pregnancy and peak at day 12 of pregnancy with a 7 fold increase in galanin levels from the non-pregnant levels (Vrontakis et al., 1992), consistent with galanin directly

regulating alveolar development during early pregnancy. $Gal^{-/-}$ mice are able to lactate following a second pregnancy, indicating that lobuloalveolar development is delayed rather then completely disrupted in these mice, supporting a developmental lag in $Gal^{-/-}$ mice. While the specific role of each of the galanin receptor subtypes during mammary development is unknown, mice with a null mutation of Galr1 demonstrate normal ductal side branching and lactogenesis (MJN, CJO, A. S. Jacoby and TPI, unpublished data), demonstrating that Galr1 is not essential for galanin-controlled lactogenesis, or prolactin regulation.

Galanin exerts its activity in a direct endocrine manner and via modulation of prolactin secretion. Both mechanisms must contribute to complete development because although prolactin treatment of $Gal^{-/-}$ mice can rescue lactation, this rescue is incomplete and a subset of alveoli remain undifferentiated.

Targeted disruption of components of the prolactin signalling cascade have demonstrated the importance of this pathway in mammopoiesis and lactation (Horseman et al., 1997; Liu et al., 1997; Ormandy et al., 1997). Likewise, we have also recently demonstrated that absolute levels of positive and negative modulators of the prolactin pathway are essential for normal mammary development (Lindeman et al., 2001). In this study we have shown that galanin can act directly on the mammary gland to induce many of the same genes induced by prolactin, an effect not simply due to transcriptional regulation of prolactin or prolactin receptor (data not shown). This indicates that like prolactin, galanin induced signalling is probably an early event in the regulation of mammary epithelial cell proliferation and differentiation. Galanin was able to induced transcription of several genes which have a demonstrated role in mammary gland

development including GHR, IGF-1, osteopontin, Id2 and Elf5. GHR and IGF-1 have well documented roles in the regulation of ductal growth and milk protein expression (X) with several studies suggesting that IGF-1 is a downstream target of growth hormone signalling (X). Galanin also has a neuroendocrine role, regulating pituitary growth hormone (Chan et al., 1996) identifying that galanin regulates growth hormone action at the level of both ligand and receptor.

Members of the intergrin signaling family are also essential for mammary gland development (X). Osteopontin a secreted intergrin binding protein that is implicated in the progression of breast cancer (Tuck and Chambers, 2002) was up-regulated by galanin and prolactin treatment. Transgenic mice expressing osteopontin antisense RNA have reduced lobuloalveolar development and fail to lactate (Nemir et al., 2000).

Both galanin and prolactin induced transcription of the epithelial specific *ets* transcription factor Elf5. We have recently identified Elf5 as a prolactin receptor regulated gene by transcript profiling of mammary transplants devoid of prolactin receptor (Harris et al.). Further, we have demonstrated that Elf5 is critical for both embryonic and mammary gland development (Zhou et al.) identifying that Elf5 maybe a critical mediator of galanin and prolactin signalling.

Galanin treatment also resulted in activation of Stat5 a transcription factor essential for lobuloalveolar development (Liu et al., 1997). Stat5 is also activated by prolactin receptor, growth hormone receptor and epidermal growth factor receptor in the mammary gland (Gallego et al., 2001), as well as having decreased binding activity in the mammary glands of $ID2^{-/-}$ mice (Mori et al., 2000). Galanin also activates another Stat family member (Stat3) in the dorsal root ganglion (D. Wynick, data not shown).

While galanin and prolactin regulated many of the same genes, differences in gene regulation occurred. Interestingly, galanin treatment alone could not induce activation of the MAP kinase signalling pathway but prolactin or prolactin + galanin resulted in marked specific activation of ERK. MAP kinase pathway signaling has an established role in the regulation of cell proliferation. This may explain why IAH + galanin was sufficient to induce epithelial cell differentiation but not proliferation, whereas increased proliferation of epithelial cells was evident in those mammary glands exposed to prolactin.

In this study we have demonstrated several novel functions of galanin during mammary gland development. Galanin acts directly on the mammary gland in an endocrine manner and as a positive regulator of prolactin signalling via regulation of pituitary prolactin secretion. These results establish galanin a neuropeptide critical for neuronal development and function as a hormone essential for mammary gland development.

Materials and methods

Animals

The development of mice with a null mutation of the galanin gene has been described (Wynick et al., 1998). $Gal^{-/-}$ mice used in these studies were of the 129OlaHsd genetic background. $Rag1^{-/-}$ mice (Mombaerts et al., 1992) on the inbred C57BL/6J background were purchased from Animal Resource Centre, Perth, Australia. All animals were specific pathogen free and housed with food and water *ad libitum* with a 12 hr day/night cycle at 22^{O}C and 80% relative humidity.

mRNA Isolation

The 4th inguinal mammary gland was frozen in liquid nitrogen before storage at -80^{O}C prior to use. Total RNA was extracted using TRIZOL Reagent (Gibco BRL) according to the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

First strand cDNA synthesis used avian myeloblastosis transcriptase (Promega) according to the manufacturer's instructions. PCR primers for Galanin (Acc No. NM 010253), Galr1 (Acc No. NM 008082), Galr2 (Acc No. NM 010254), Galr3 (Acc No. NM 015738) and GAPDH (Acc No. M32599) were designed on the basis of mismatch to other genes. The following primers were used in this study: (Galanin) mGal-F1 5'-TGCAGTAAGCGACCATCCAG-3' (forward) and mGal-R1 5'-AGCACAGGACACACGTGCAC-3' (reverse), (Galr1) mGalr1-F1 5'-CGCCTTCATCTGCAAGTTTA-3' (forward) and mGalr1-R1 5'-

CAGGACGGTCTGTGCAGT-3' (reverse), (Galr2) mGalr2-F1 5'-TGCCTTTCCAGGCCACCATC-3' (forward) and mGalr2-R1 5'-GCGTAAGTGGCACGCGTGAG-3' (reverse), (Galr3) Galr3-F1 5'-CCTGGCTCTTTGGGGCTTTCGTG-3' (forward) and Galr3-R1 5'-AGCGCGTAGAGCGCGGCCACTG-3' (reverse), (GAPDH) GAPDH-F1 5'-TGACATCAAGAAGGTGGTGAAGC-3' (forward) and GAPDH-R1 5'-AAGGTGGAAGAGTGGGAGTTGCTG-3' (reverse). The amplification regime consisted of a 94°C 10 min denaturation cycle, followed by 94°C for 25 sec, 58°C for 30 sec, and 72°C for 2 min, for 33 cycles. An elongation step of 72°C for 5 min ended the PCR.

Oligonucleotides for internal hybridisation of PCR products were 5'-AATGGCCACGTAGCGATCCA-3' (Galr1), 5'-GTAGCTGCAGGCTCAGGTTCC-3' (Galr2) and 5'-GTGGCCGTGGTGAGCCTGGCCT-3' (Galr3).

Recombined mammary gland transplantation

Donor mammary tissue (1 mm^3) from $Gal^{+/+}$ or $Gal^{-/-}$ 12 week old mice was inserted into the excised fat pad of $Gal^{+/+}$ or $Gal^{-/-}$ 3 week old mice cleared of endogenous epithelium. This recombined mammary epithelium-stroma complex was then grafted between the abdominal cavity and skin, between the 3rd and 4th mammary glands of 3 week old $Rag1^{-/-}$ mice (Brisken et al., 1998). This procedure resulted in 100% transplant survival with >95% showing ductal outgrowth. Using this method, recombinations of mammary epithelium and stroma were produced that allowed deletion of the galanin gene from stroma and/or epithelium.

Histological analysis

Mammary whole mounts were made by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining overnight. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 min and storage and photography in methyl salicylate (Bradbury et al., 1995). Morphometric analysis was performed by counting the number of side branches, alveolar buds or lobulo-alveoli per mammary gland (n=5) for explant cultures or from 4 representative fields of view from whole 4th inguinal glands.

Prl treatment of mice

On the morning of the observation of a vaginal plug, 6-8 week old mice were implanted with a 0.25 μl per hour, 28 day mini-osmotic pump (Alzet) containing either unmodified Prl prepared as described (Chen et al., 1998). Either 0.6 or 1.2 μg were delivered per 24 hr. On the first day post-partum maternal behaviour of mothers was observed, pups were examined for the presence of milk and glands were taken for histological analysis.

Mammary explant culture

Four week old BALB/c mice were implanted with estrogen, progesterone and cholesterol pellets (Ginsburg and Vonderhaar, 2000). Following nine days of treatment, the #4 glands were removed and stretched onto siliconized lens paper and placed into petri dishs containing 2 mL of Waymouths 152/1 medium supplemented with penicillin (100 U/ml),

streptomycin (100 μg/ml), gentamycin sulfate (50 μg/ml), 20 mM HEPES, insulin (5 μg/ml), hydrocortisone (100 ng/ml) and aldosterone (100 ng/ml) to monitor ductal side branching, with and without 100 nM rat galanin (Auspep). To assess lobuloalveolar development ovine Prl (Sigma, 1 μg/ml) was added to the medium. Glands were maintained in a tri-gas incubator at 50% O_2 and 5% CO_2 in air. Medium was changed after 24 hr, then every second day for 6 days before morphology and histology were assessed.

Transcript profiling

Total RNA was extracted using TRIZOL Reagent (Gibco BRL), purified using RNeasy Mini Kit (QIAGEN), cDNA synthesis performed using Superscript II (Invitrogen Life Technologies), synthesis of Biotin-labeled cRNA performed using BioArray HighYield RNA Transcript labeling Kit (Enzo Diagnostics) and hybridised to Affymetrix MGU74v2 GeneChips overnight as per manufacturer's instructions. Arrays were performed in duplicate using 4-6 glands per treatment group from two separate replicate experiments. Analysis was performed using the Affymetrix GeneChip v5 software, with treatment groups compared back to IAH treatment as the baseline comparison.

Quantitative RT-PCR

Quantitative PCR was performed using LightCycler technology (Roche). PCR reactions were performed in 10 μL volume with 1 μL of cDNA, 5 pmoles of each primer and FastStart DNA Master SYBR Green I enzyme mix (Roche) as per manufacturers instructions. Relative quantitation of the product was performed by comparing the

crossing points of different samples normalised to an internal control (β-Actin). Each cycle in the linear phase of the reaction corresponds to a two fold difference in transcript levels between samples. Each reaction was performed in triplicate using pooled RNA from the 4-6 mammary glands or the treatment groups utilised for transcript profiling.

Western analysis

Following RNA extraction from mammary glands using TRIZOL Reagent (Gibco BRL), protein was extracted following the manufacturer's instructions. Protein was separated using SDS-PAGE (Bio-Rad Laboratories), transferred to PVDF (Millipore) and blocked overnight with 5% skim milk powder, 2% fetal bovine serum, 50 mM sodium phosphate, 50 mM NaCl and 0.1% Tween 20. Membranes were incubated with one of the following primary antibodies: α-milk protein (Accurate Chemical & Scientific Corporation), α-STAT5a (Upstate Biotech), α-phospo-STAT5, α-phospho-Erk1/2, α-Erk2, α-phospho-AKT (S472), α-phospho-AKT (T308), α-AKT (Cell Signaling Technology) or α-β-Actin (Sigma). 20 μg of protein was loaded per lane except for α-milk protein were 400 ng of protein was loaded. Specific binding was detected using Horseradish peroxidase conjugated secondary antibodies (Amersham Biosciences) with Chemiluminescence Reagent (PerkinElmer) and Biomax Light Film (Eastman Kodak Company).

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Figure legends

Figure 1.

Galanin and galanin receptor expression in the mammary gland. Expression of galanin and galanin receptors at various developmental stages of mammary gland development by RT-PCR and hybridization of RT-PCR products with an internal oligonucleotide (Galr1-3). Developmental stages are virgin mice at estrous (est.), virgin mice at diestrous (diest.), days 7, 12 and 16 of pregnancy (7D, 12D & 16D pregnant), lactation and 5 days of involution (5D invol.).

Figure 2.

Galanin is essential for mammary gland development. (A-B) Carmine stained whole mounts of 4th mammary glands at (A) day 12 of pregnancy and (B) 1st day post-partum. (C) Haematoxylin and eosin stained 5 μm sections from mammary glands at 1st day post-partum. (D) Examination of milk protein (WDMN-1, β-casein and WAP) mRNA expression by quantitative RT-PCR at the 1st day post-partum. Fold difference in expression levels expressed as $Gal^{-/-}$ verse $Gal^{+/+}$. (E) Examination of milk protein (WDMN-1, β-casein and WAP) mRNA expression $Gal^{-/-}$ treated with prolactin verse $Gal^{+/+}$. (F) Analysis of lactation in $Gal^{+/+}$, $Gal^{-/-}$ and $Gal^{-/-}$ mice treated with prolactin throughout pregnancy. Mammary glands from $Gal^{-/-}$ mice have arrested development compared to wild type littermates. Alveolar proliferation is inhibited at mid pregnancy (A) with a failure of full lobuloalveolar development at the 1st day post-partum (B). Alveoli from $Gal^{-/-}$ mammary glands are less differentiated (C) lactating ducts are clear

and open, while non-lactating less differentiated ducts retain colostrum (contents staining pink with oil drops) and have reduced milk protein gene expression (D). $Gal^{-/-}$ mice treated with prolactin have lactation restored (F) however, lobuloalveoli fail to fully differentiate, indicated by the presence of less differentiated alveoli (C) and failure of milk protein gene expression (E).

Figure 3.

Galanin does not act via autocrine or paracrine mechanisms to regulate mammary gland development. Carmine stained whole mounts of $Gal^{-/-}$ (A,C) & $Gal^{+/+}$ (B,D) epithelium transplanted into the fat pad of $Rag1^{-/-}$ mice cleared of endogenous epithelium. (A,B) virgin (C,D) 1st day post-partum. Inserts haematoxylin and eosin stained 5 μm sections from the same glands. Deletion of galanin gene from the epithelium does not effect normal mammary gland morphology or histology.

Figure 4.

Galanin acts directly on the mammary gland to induce lobuloalveoli development. (W/Mount) whole mounts of mammary glands following whole organ culture *in vitro* after culture in the presence of insulin, aldosterone and hydrocortisone (IAH), with or without galanin and prolactin as indicated. Arrows indicate lobuloalveoli. (H&E) haematoxylin and eosin stained 5 μm sections from the same glands. Western blot analysis of the expression of milk proteins, STAT5, ERK and AKT in mammary glands following IAH, + galanin and/or prolactin treatment. Milk protein (α-casein, β-casein and WAP) expression in explant mammary glands demonstrates that milk protein levels are

increased following galanin and prolactin, prolactin or galanin treatment alone. Increased levels of phosphorylated Stat5 was observed in mammary glands following treatment with galanin and/or prolactin. Galanin alone was not able to induce activation of the MAP kinase pathway. Phosphorylated ERK1/2 was increased in mammary glands treated with prolactin or prolactin + galanin despite a decrease in the total levels of ERK. This demonstrates marked specific activation of MAP kinase signalling in those glands treated with prolactin. Examination of the PI3 kinase pathway revealed decreased mobility but no increase in total AKT in explants receiving prolactin. This decrease in mobility was not due to phosphorylation of the two residues most commonly associated with AKT activation.

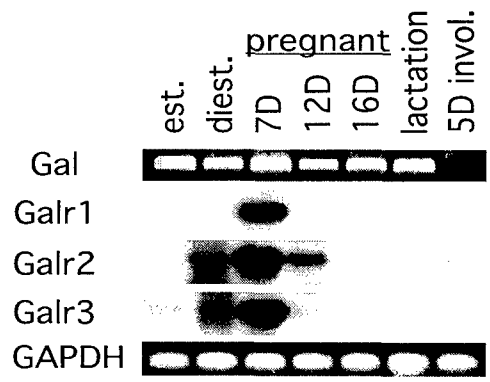
Figure 5.

Transcriptional response of the mammary gland to galanin and prolactin. Transcript profiling of whole mammary gland explants. (A) Venn diagram showing the total number of genes found to be increasing or decreasing in response to galanin and prolactin. (B) Response of selected genes to IAH + galanin (G), + prolactin (P) or + prolactin and galanin (PG) treatment. IAH + galanin treatment alone is sufficient to induce mammary epithelial cell differentiation as demonstrated by induction of milk protein gene expression.

Figure 6.

Summary of the role of galanin in mammary gland development. The stages of mammary gland development are shown schematically with causative reproductive

events indicated above and descriptions of subsequent morphological changes given above each dashed arrow. Hormone secretion is shown by solid arrows. Regulatory influences on hormones or morphology are indicated by dashed lines that are positive (arrow heads) or negative (lines).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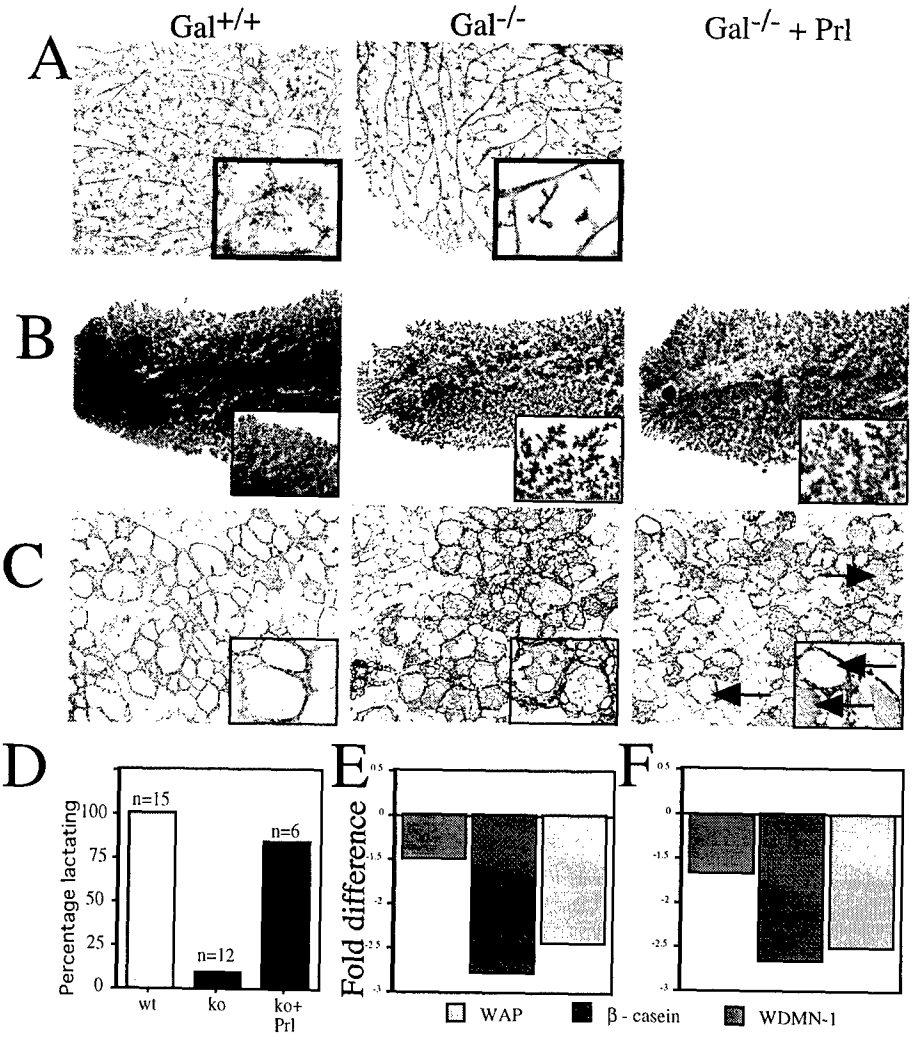
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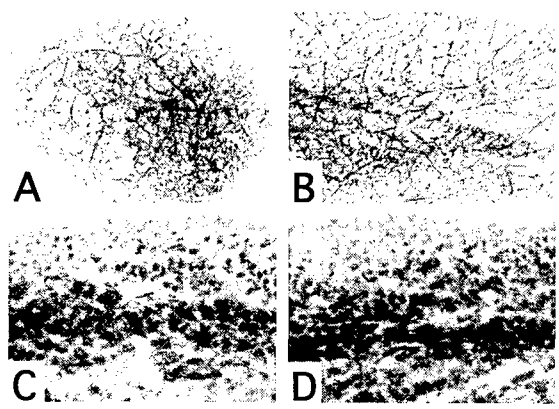
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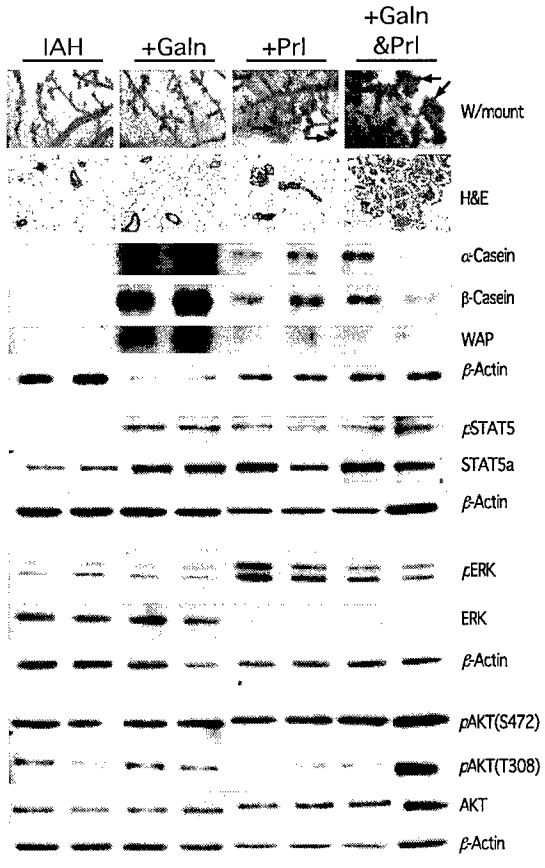
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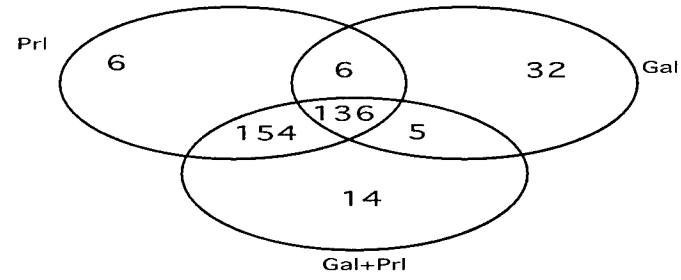
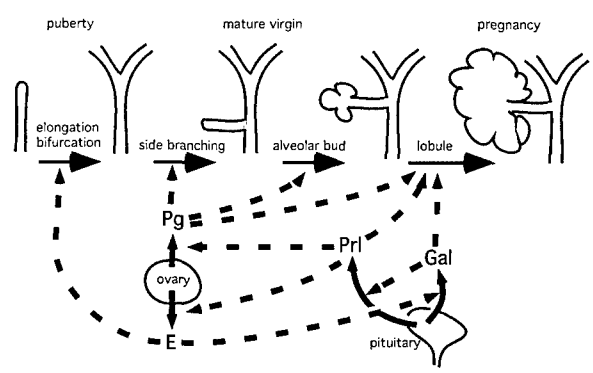


B

Accession #	Title	Symbol	Annotation	Fold change			Q. RT-PCR		
				PG	G	P	PG	G	P
PG.G.P									
V00856	whey acidic protein	Wap	milk protein	171	282	251	57	289	126
M93428	glycosylation depend. cell adhes. mol. 1	Glycam1	cell adhesion	86.2	101	133	74	541	257
M36780	casein alpha	Csna	milk protein	73.5	113	91.8			
D10215	casein gamma	Csng	milk protein	58.1	115	83.9			
X04490	casein beta	Csnb	milk protein	41.4	48.8	46.2	100	600	190
M87863	alpha-lactalbumin		milk protein	28.6	77.2	31.1			
M10114	kappa-casein		milk protein	26.4	33.4	30.7			
V00740	casein delta	Csnd	milk protein	14.1	45.3	41.6			
X60367	cellular retinol binding protein I	CRBPI	retinol metabolism	7.41	5.7	7.11			
AF049702	E74-like factor 5	Elf5	transcription factor	6.19	7.11	7.26	7.1	29.4	9.1
X93037	WDMN-1	Expi	milk protein	5.78	10.6	7.41	4	19	17
X13986	secreted phosphoprotein 1	Spp1	cell adhesion	3.86	10.8	6.02	-1.5	10.3	2.6
M31680	growth hormone receptor	Ghr	GH signaling	3.66	1.51	2.87	6.01	3.01	4.26
L12447	IGF binding protein 5	Igfbp5	IGF signaling	3.07	3.66	3.51			
M38337	milk fat globule-EGF factor 8	Mfge8	cell adhesion	2.81	4.99	3.94			
X95279	spot14	SP14	lipogenesis	2.53	1.34	2.87	1.2	1.52	3.4
M81445	connexin 26	Cx26	gap junction	2.38	2.04	2.35			
AF077861	helix-loop-helix protein Id2	Id2	transcription factor	1.79	1.94	1.99			
X16490	plasminogen activator inhibitor 2	PAI-2	protease inhibitor	-5.8	-3.5	-4.4			
M33960	plasminogen activator inhibitor 1	PAI-1	protease inhibitor	-4.1	-1.6	-2.6			
D10837	ras recision	rrg	tumor-suppressor	-2.5	-1.5	-1.9			
PG.P not G	gene								
M19681	PDGF-inducible protein (JE) gene	JE	chemoattractant	2.38	-1.3	2.69			
U03419	procollagen, type I, alpha 1	Col1a1	cell adhesion	2.33	-1.2	2.31			
D29678	cyclin-dependent kinase 5	Cdk5	cell cycle	1.43	1.16	2.23			
X58251	procollagen, type I, alpha 2	Col1a2	cell adhesion	2.19	-1.4	2.16			
L42115	solute carrier family 1, member 7	Slc1a7	transport	2.01	-1.1	2			
Y07688	nuclear factor I/X	Nfix	transcription factor	1.73	1.13	1.87			
U82758	claudin 5	Cldn5	tight junction	2.08	-1	1.85			
AI154710	zinc finger protein 125	Zfp125	DNA binding	-2.2	1.1	-6.1			
D50311	myocyte enhancer factor 2B	Mef2b	transcription reg.	-2.5	-1	-2			
G not P not PG									
AV295044	glucose phosphate isomerase 1 complex	Gpi1	glycolysis	-1.5	1.7	-1.6			
AA717826	dermatopontin	Dpt	cell adhesion	1.29	2.6	1.32			
X81584	IGF binding protein 6	Igfbp6	IGF signaling	1.79	-2.3	1.43			
M57683	PDGF receptor alpha	Pdgfra	Tyr kinase receptor	1.08	-2	1.2			
P not PG not G									
L19932	beta ig-h3		cell adhesion inhib.	-1.1	-1.3	1.91			
J02872	granzyme G	Gzmg	proteolysis	-1.2	1.02	-2.5			
PG not P not G									
X04367	PDGF receptor beta	Pdgfrb	Tyr kinase receptor	1.96	-1.5	-1.2			
L32838	interleukin 1 receptor antagonist	IL-1ra	ILR1 signaling	1.93	-2.5	1.17			
D38046	topoisomerase (DNA) II beta	Top2b	DNA metabolism	1.84	1.05	-1.2			
X83934	ryanodine receptor 3	Ryr3	calcium homeostasis	-2.1	-1.1	1.01			
AV362816	steroidogenic acute regulatory protein	Star	steroid hormone biosyn	-1.9	1.02	-1			


puberty
mature virgin
pregnancy
elongation
bifurcation
side branching
alveolar bud
lobule
Pg
Prl
Gal
ovary
E
pituitary

Manuscript in preparation

Galanin controls mammary gland development via regulation of prolactin phosphorylation

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Abstract

Galanin has an established role in neuronal and neuroendocrine regulation and was recently demonstrated to regulate mammary gland development by both direct and indirect endocrine mechanisms. Galanin knockout mice display reduced prolactin levels and lactation failure, which prolactin treatment rescues. Prolactin is a polypeptide pituitary hormone essential for mammary gland development and lactation. Several post-translational modified forms of prolactin exist that are the result of glycosylation, truncation and phosphorylation that may have an effect on the physiological function of prolactin. We report that in addition to regulating prolactin secretion galanin also regulates pituitary prolactin phosphorylation. Treatment of wildtype mice with a molecular mimic of phosphorylated prolactin (S179D) inhibited lactation and lobulo-alveolar development. S179D treatment resulted in a decrease in activated Stat5 the major downstream signaling pathway induced by prolactin. Transcript profiling of wildtype, S179D treated and galanin knockout mice at the 1st day post-partum identified groups of genes similarly regulated between S179D and galanin knockout mice. These data identify a unique regulatory mechanism whereby galanin regulates prolactin phosphorylation to modify prolactin function.

Introduction

Prolactin is a polypeptide hormone that is essential for mammary gland development and lactation (X). Mammary transplantation studies using prolactin receptor (Prlr) knockout mice have demonstrated that epithelial Prlr is essential for lobuloalveolar development and has identified various downstream targets of Prlr signaling (X). Prolactin is synthesised by mammary epithelial cells during pregnancy and lactation and may act via autocrine or paracrine mechanism (X). However, the anterior pituitary is the principle site of prolactin synthesis and almost all of prolactins functions are attributed to pituitary prolactin (X). The importance of the prolactin signaling in mammary gland development is further demonstrated by the essential requirement of both positive and negative regulators of prolactin signaling for normal mammopoiesis (X). Prolactin signaling is regulated at the level of both pituitary prolactin synthesis and secretion, as well as Prlr signaling (X). In addition to these regulatory mechansims prolactin also undergoes several post-translational modifications that include proteolytic cleavage, phosphorylation and glycosylation (X). In general these modifications result in lower biological activity of prolactin and therefore serve as another mechanism to regulate the biological function of prolactin.

Galanin is a neuropeptide that has a wide range of reported biological functions including neuronal development and neuroendocrine regulation (X). Galanin is a paracrine growth factor for the prolactin secreting lactotroph (X). Overexpression of galanin in the lactotroph induces hyperprolactinemia (X), while galanin knockout mice display reduced prolactin levels (X). These data demonstrate that galanin is a positive regulator of pituitary prolactin secretion. We have also recently shown that galanin is

essential for mammary gland development (X). Galanin knockout mice fail to lactate which is rescued by prolactin treatment demonstrating an indirect neuroendocrine role (X). However, despite rescuing lactation prolactin does not fully restore epithelial cell differentiation. Galanin and galanin receptors are expressed in the mammary gland and galanin also acts directly on the mammary gland to induce epithelial cell differentiation (X). Thus, galanin regulates mammary gland development by multiple mechanisms.

In this study we report that prolactin phosphorylation is increased in the pituitaries of galanin knockout mice. Using a molecular mimic of phosphorylated prolactin (S179D) the role of phosphorylated prolactin during pregnancy and lactation was examined. S179D prolactin inhibited lactation and lobuloalveolar development in mice, an effect associated with inhibition of Stat5 activation. Transcript profiling identified groups of genes similarly regulated between S179D prolactin treated and galanin knockout mice during late pregnancy. These data identify a novel regulatory mechanism where galanin a positive regulatory of prolactin secretion also inhibits prolactin phosphorylation which reduces the biological functions of prolactin in the mammary gland.

Results

The phosphorylation state of Prl is altered in galanin knockout mice

Galanin has an established role in the modulation of the release of a number of hormones including Prl, a hormone crucial for mammary development and lactogenesis. Recently, different biological functions have been identified *in vitro* for several modification states of Prl. We sought to determine if galanin regulates not only the release of Prl but also the modification state of Prl.

Measurement of the levels of secreted unmodified and phosphorylated Prl were undertaken using pituitaries from both $Gal^{-/-}$ and $Gal^{+/+}$ mice. Initially, female mice were used, but as galanin is extremely sensitive to estrogen regulation, varying greater then 30-fold during the estrous cycle (Fantl et al., 1988), levels varied too greatly at all stages of the estrous cycle to allow accurate measurement. In wildtype male mice 80.6±4.1% of Prl was present in the unmodified form, while 20.0±1.9% was in the phosphorylated form (Fig. 1A). $Gal^{-/-}$ mice, however, had 68.9±3.2% of Prl as the unmodified form and 31.1±2.1% as the phosphorylated form (Fig. 1B). Thus, the relative ratio of unmodified to phosphorylated Prl was 4:1 in wildtype mice, compared to 2:1 in knockout mice (Fig. 1, $p<0.0001$ Students (unpaired) T-test).

Phosphorylated Prl inhibits lobuloalveolar development and prevents lactation

We next investigated whether altering the ratio of phosphorylated to unphosphorylated Prl had an effect on lobulo-alveolar development or lactation. $Gal^{+/+}$ mice were treated

during pregnancy with S179D Prl (Chen et al., 1998), a molecular mimic of phosphorylated Prl. S179D Prl treatment of $Gal^{+/+}$ mice inhibited lactation (Fig. 2A) and resulted in the death of all pups within 24 hrs of birth. Despite continued suckling, the stomachs of pups from S179D Prl-treated mice did not contain milk. Morphological examination of the 4th mammary gland at the first day post-partum showed that lobulo-alveolar development was reduced compared to saline treated wildtype mice (Fig. 2B) and was similar to levels observed in $Gal^{-/-}$ mice (Naylor et al.). Histological examination confirmed reduced development and revealed failed lactogenesis (Fig. 2C). The failure of lobulo-alveolar development and lactation was associated with a failure of alveolar differentiation as determined by analysis of milk protein mRNA expression. WDMN-1 and β-casein mRNA levels were measured by quantitative RT-PCR. S179D prolactin treatment resulted in a xx fold decrease of WDMN-1 and xx fold decrease of β-casein compared to control mice (Fig. 2D). These data indicate that alteration in the ratio of phosphorylated to non-phosphorylated Prl can modify Prl's developmental and lactogenic activities. Treatment of $Gal^{-/-}$ mice with S179D Prl did not increase or decrease the severity of any of the morphological or lactational defects already present in these animals (Fig. 2A, data not shown).

Phosphorylated Prl inhibits STAT5 activation

Binding of Prl to the Prlr results in receptor dimerisation and subsequent activation of the Jak/ Stat pathway (XX), the major signaling pathway utilised by Prlr. Both Jak2 and Stat5a have been demonstrated as essential for mammary gland development and milk protein gene expression (XX). Activation of Prlr can also result in the induction of the

MAPK and PI3K signaling pathways (X). We next investigated if treatment of mice with S179D Prl resulted in the inhibition of Prlr signaling pathways in the mammary gland by Western analysis.

The levels of phosphorylated and total ERK did not change between saline and S179D treated mice indicating that signaling via the MAPK pathway is not effected by prolactin phosphorylation (Fig. 3C). Likewise, the amount of total and phosphorylated Akt/ PKB a downstream target of the PI3K signaling pathway also did not significantly change between the two groups of mice (Fig. 3D). The levels of Stat5a were equal in the mammary glands of saline and S179D treated mice (Fig. 3A), however the amount of phosphorylated Stat5 was markedly reduced in the mammary glands of mice treated with S179D prolactin (Fig. 3A). These data indicate that phosphorylated Prl inhibits the lactogenic function of Prl by inhibiting activation of the Jak/Stat pathway.

The transcriptional response of the mammary gland to S179D treament is both similar and different to Gal$^{-/-}$ mice at lactation

To further investigate if control of prolactin phosphorylation is a regulatory mechanism utilised by galanin we transcript profiled mammary glands from wildtype, S179D treated, Gal$^{-/-}$ and Gal$^{-/-}$ rescued with Prl mice on the first day post-partum. Affymetrix MGU74v2 genechips were used which contain a total approximately 12000 genes comprising of 9000 known genes and 3000 Ests. The total number of genes found either increasing or decreasing compared to wild type mice was determined using Affymetrix GeneChip v5 software (MAS 5). This analysis (Fig. 5) was validated using quantitative RT-PCR and

similar results were also found using ANOVA, ranking by the coefficient (data not shown).

The Venn diagrams (Fig. 4) demonstrate that a number of genes where positively or negatively regulated by the addition of S179D prolactin .....

Together these data show that galanin regulates prolactin phosphorylation which is a negative regulator of prolactin signalling. Phosphorylation of prolactin inhibtis activation of the Jak/ Stat pathway which results in inhibition of lobulalveolar differentiation and lactogenesis.

Discussion

Materials and methods

Animals

The development of mice with a null mutation of the galanin gene has been described (Wynick et al., 1998). $Gal^{-/-}$ mice used in these studies were of the 129OlaHsd genetic background. All animals were specific pathogen free and housed with food and water *ad libitum* with a 12 hr day/night cycle at 22^{o}C and 80% relative humidity.

mRNA Isolation

The 4th inguinal mammary gland was frozen in liquid nitrogen before storage at -80°C prior to use. Total RNA was extracted using TRIZOL Reagent (Gibco BRL) according to the manufacturer's instructions.

Histological analysis

Mammary whole mounts were made by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining overnight. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 min and storage and photography in methyl salicylate (Bradbury et al., 1995).

Two-dimensional polyacrylamide gel electrophoresis

Following decapitation the anterior pituitary was removed, cut into 1mm pieces, rinsed in PBS to remove material from damaged cells and incubated in DMEM containing 0.1% bovine serum albumin for 2 hr at 37°C in an atmosphere of water-saturated 5% CO_2. At the end of the 2 hr incubation period, the medium was removed and frozen prior to preparation for two-dimensional gel analysis. Two pituitaries were used per 2 mL of incubation medium to allow sufficient Prl accumulation in the samples from the $Gal^{-/-}$ mice. The protein in the incubation medium were precipitated in 4 volumes of -20°C acetone overnight, collected by centrifugation and then dissolved in urea lysis buffer containing 9M urea, 5% 2-mercaptoethanol, 4% ampholines pH 4-6.5 (Sigma). Electrophoresis was performed according to the method of Ho *et al.* (Ho et al., 1993). After electrophoresis the gel was silver stained (Oakely et al., 1980) and the spots were

identified by reference to standards as described previously (Oetting and Walker, 1985) and by reference to a co-run sample that was subject to western blot analysis (Oetting and Walker, 1985). Spot intensity was analyzed using a Kodak image analysis system (Eastman Kodak Co.).

Phosphorylated and unmodified Prl treatment of mice

On the morning of the observation of a vaginal plug, 6-8 week old mice were implanted with a 0.25 μl per hour, 28 day mini-osmotic pump (Alzet) containing either unmodified Prl or the molecular mimic of phosphorylated Prl S179D, both hormones prepared as described (Chen et al., 1998). Either 0.6 or 1.2 μg were delivered per 24 hr. On the first day post-partum maternal behaviour of mothers was observed, pups were examined for the presence of milk and glands were taken for histological analysis.

Transcript profiling

Total RNA was extracted using TRIZOL Reagent (Gibco BRL), purified using RNeasy Mini Kit (QIAGEN), cDNA synthesis performed using Superscript II (Invitrogen Life Technologies), synthesis of Biotin-labeled cRNA performed using BioArray HighYield RNA Transcript labeling Kit (Enzo Diagnostics) and hybridised to Affymetrix MGU74v2 GeneChips overnight as per manufacturer's instructions. Arrays were performed in duplicate using 4-6 glands per treatment group from two separate replicate experiments. Analysis was performed using the Affymetrix GeneChip v5 software, with treatment groups compared back to IAH treatment as the baseline comparison.

Quantitative RT-PCR

Quantitative PCR was performed using LightCycler technology (Roche). PCR reactions were performed in 10 μL volume with 1 μL of cDNA, 5 pmoles of each primer and FastStart DNA Master SYBR Green I enzyme mix (Roche) as per manufacturers instructions. Relative quantitation of the product was performed by comparing the crossing points of different samples normalised to an internal control (β-Actin). Each cycle in the linear phase of the reaction corresponds to a two fold difference in transcript levels between samples. Each reaction was performed in triplicate using pooled RNA from the 4-6 mammary glands or the treatment groups utilised for transcript profiling.

Western analysis

Following RNA extraction from mammary glands using TRIZOL Reagent (Gibco BRL), protein was extracted following the manufacturer's instructions. Protein was separated using SDS-PAGE (Bio-Rad Laboratories), transferred to PVDF (Millipore) and blocked overnight with 5% skim milk powder, 2% fetal bovine serum, 50 mM sodium phosphate, 50 mM NaCl and 0.1% Tween 20. Membranes were incubated with one of the following primary antibodies: α-STAT5a (Upstate Biotech), α-phospo-STAT5, α-phospho-Erk1/2, α-Erk2, α-phospho-AKT (S472), α-AKT (Cell Signaling Technology) or α-β-Actin (Sigma). 20 μg of protein was loaded per lane. Specific binding was detected using Horseradish peroxidase conjugated secondary antibodies (Amersham Biosciences) with Chemiluminescence Reagent (PerkinElmer) and Biomax Light Film (Eastman Kodak Company).

Acknowledgments

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Figure legends

Figure 1.

Levels of Prl isoforms in pituitaries of $Gal^{-/-}$ mice. Stained 2-D gel analysis of anterior pituitary Prl isoforms (U-unmodified Prl, P-phosphorylated Prl) from $Gal^{+/+}$ (A) and $Gal^{-/-}$ mice (B).

Figure 2.

A molecular mimic of phosphorylated Prl inhibits lobulo-alveolar development and lactation in mice. (A) Analysis of lactation in $Gal^{+/+}$, $Gal^{-/-}$ and $Gal^{-/-}$ mice treated with prolactin, $Gal^{+/+}$ treated with S179D and $Gal^{-/-}$ mice treated with S179D throughout pregnancy. (B) Carmine stained whole mount analysis of #4 mammary glands. (C) Haematoxylin and eosin stained 5 μm sections from the same glands. In $Gal^{+/+}$ mice treated with S179D lobuloalveolar proliferation was decreased and lobuloalveolar differentiation not complete as lactation failed. Arrows indicate ducts, lactating ducts are clear, while non-lactating less differentiated ducts retain colostrum (contents staining pink with oil drops). (E) Examination of milk protein (WDMN-1, β-casein and WAP) mRNA expression $Gal^{+/+}$ treated S179D verse $Gal^{+/+}$. Mammary epithelial cell differentiation is impaired in S179D treated mice as demonstrated by a reduction in milk gene expression.

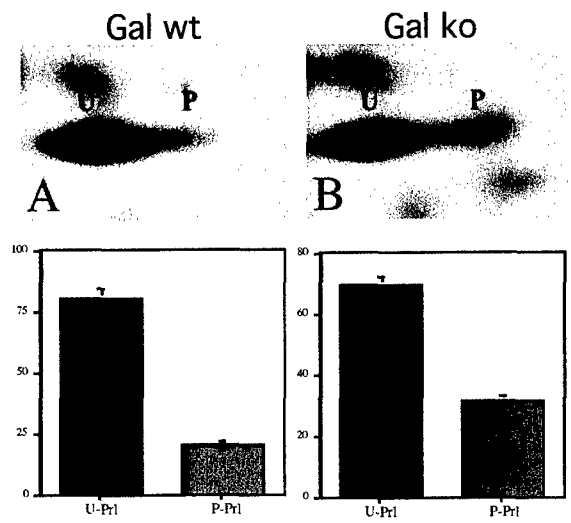
Figure 3.

Investigation of the effect of S179D on signaling pathways in the mammary gland. Western analysis of signalling pathways induced in mammary glands of saline and

S179D prolactin treated wild type mice. The levels of phosphorylated and total ERK, cyclin D1 and AKT did not change between saline and S179D treated mice indicating that signaling via the MAP kinase, PI3 kinase and the cell cyle regulator cyclin D1 is not effected by prolactin phosphorylation. The levels of Stat5a were equal in the mammary glands of saline and S179D prolactin treated mice. However the amount of phosphorylated Stat5 was markedly reduced in S179D prolactin treated mice demonstrating that phosphorylated prolactin inhibits activation of the Jak/Stat pathway.

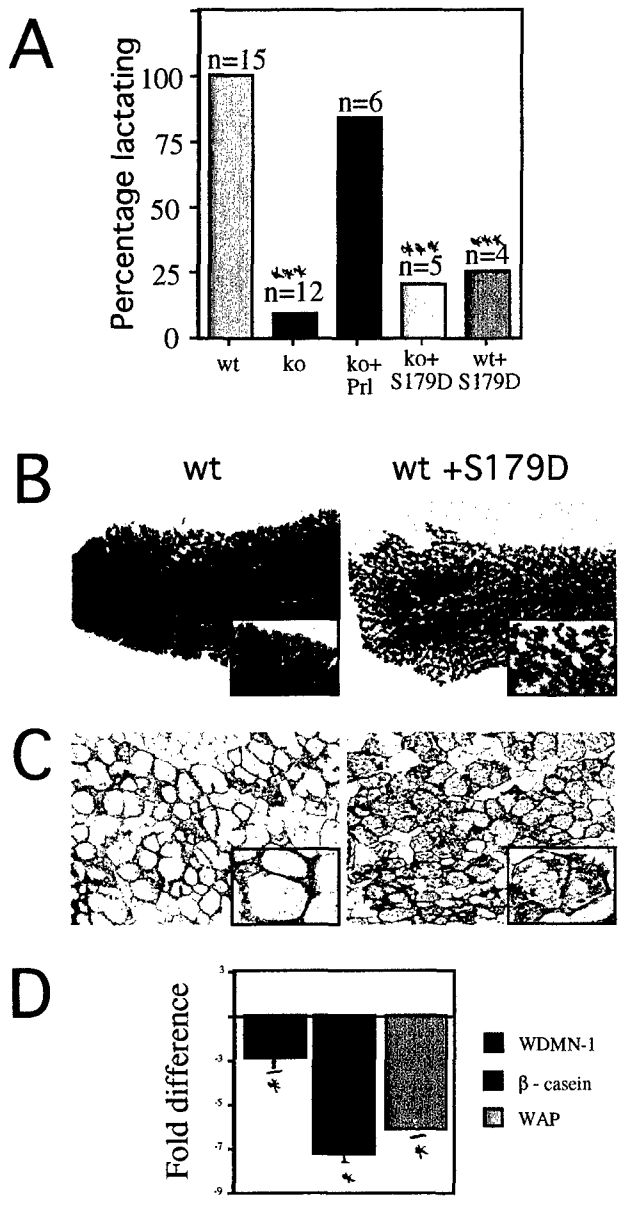
Figure 4.

Transcriptional response of the mammary gland to galanin and phosphorylated prolactin at lactation. Transcript profiling of whole mammary glands from wildtype, galanin knockout, galanin knockout + prolactin and wildtype + S179D mice at the 1st day post partum. (A) Venn diagram showing the total number of genes found to be increasing (B) Venn diagram showing the total number of genes found to be decreasing. (C) Table of a selection of genes regulated by galanin or S179D prolactin.



4 : 1 2 : 1

Relative ratio of U-Prl to P-Prl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Percentage lactating
100
75
50
25
0
n=15
n=12
n=6
n=5
n=4
wt
ko
ko+ Prl
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wt+ S179D
B
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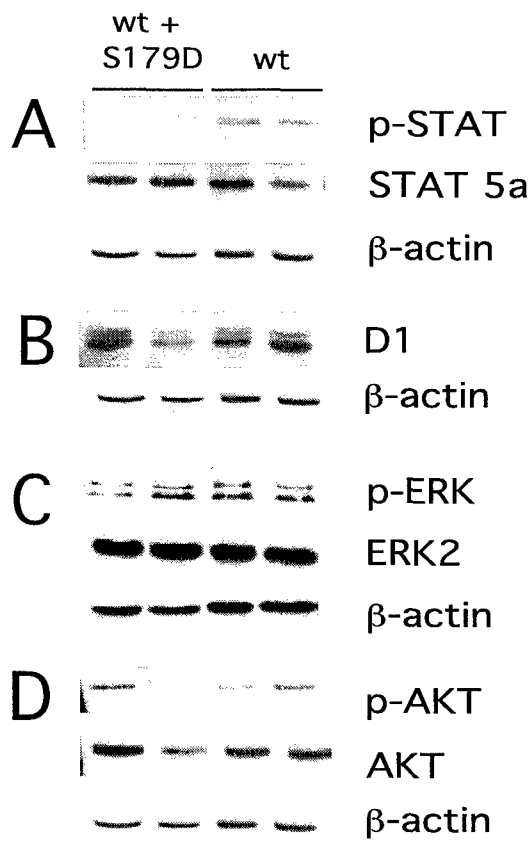
D
Fold difference
WDMN-1
β - casein
WAP


wt +
S179D
wt
A
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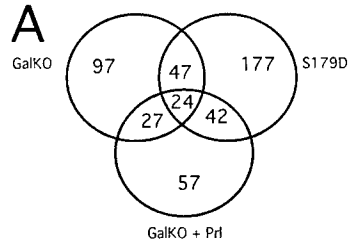
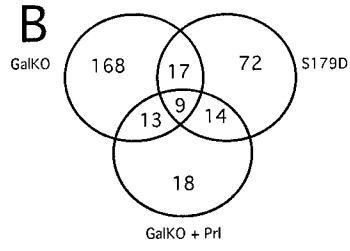


B



C

Table of regulated genes with annotation and realtime PCR

Generalised liner model

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF

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MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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FOR THE COMMANDER:

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PHYLIS M. RINEHART
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